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ORIGINAL PAPER

# Natural Occurrence, Exposure Assessment & Risk Characterization of *Alternaria* Mycotoxins in Apple By-Products in Argentina

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**Abstract**

Data on the natural occurrence of *Alternaria* mycotoxins in apple by-products is lacking in Argentina and the risk of exposure to these mycotoxins has not been characterized before. The levels of alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN), altertoxin-I (ATX-I), altertoxin-II (ATX-II), alternariol 3-sulfate (AOH-3-S), alternariol 3-glucoside (AOH-3-G), alternariol monomethyl ether 3-sulfate (AME-3-S), and alternariol monomethyl ether 3-glucoside (AME-3-G) were determined in clarified and cloudy apple juices, marmalades, and apple-based infant food from the Argentinean market, and the risk of exposure was characterized. Detectable levels of AME, TEN, TeA, AME-3-S and AOH-3-G were found in clarified juices, while the same mycotoxins plus AOH were found in cloudy apple juices in higher concentrations. AME, TEN, TeA and AOH-3G were detected in marmalades, and AOH, AME, TEN and TeA in apple infant food. Probabilistic exposure assessment and risk characterization were carried out for children between 6 months and 5 years old in Argentina. The highest risk of exposure affected children between 6 and 23 months from the consumption of apple infant food and mainly associated with the alternariols. Better control strategies to prevent the incorporation of *Alternaria* mouldy core into the process line and the establishment of legislation for *Alternaria* mycotoxins are needed in Argentina.

**Keywords** Infant foods · Consumer's risk · Modified mycotoxins · Alternariol · Food safety

## Introduction

Apples are susceptible to infection with fungi in the pre- and post-harvest stage, and toxigenic species from *Penicillium* and *Alternaria* are among their most frequent genera (Florian et al. 2018; Pavicich et al. 2020a, b). Apple fruits destined for apple concentrate production are usually of lower quality than those for retail, and mouldy fruit can be inadvertently introduced in the process line. A recent study (Pavicich et al. 2020a, b) found *Alternaria* toxins in the raw material for apple concentrate production in Argentina. The clarification step was key to reduce the concentration of most *Alternaria* mycotoxins, but cloudy final products, obtained without clarification, showed increased levels of these toxins.

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The apple concentrate can be destined to different apple by-products such as clear apple juice (AJ), cloudy AJ, apple marmalade, apple cider, and apple infant food, amongst others. The intake of cloudy apple products has increased lately due to a shift to less processed and more natural foods, and consumers perceive them as healthier. Regarding apple juice, Markowski et al. (2015) showed that the phenolic content of clear AJ was significantly lower than the cloudy one. However, the mycotoxicological risk associated to non-clarified products seems to be higher according to our results (Pavicich et al. 2020a, b). Studies from world regions showed the presence of *Alternaria* mycotoxins in apple and apple by-products (Delgado and Gómez-Cordovés 1998; Gotthardt et al. 2019; Puntscher et al. 2020), but no information about these mycotoxins in the Argentinean market is available.

Studies on the toxicity of the main *Alternaria* mycotoxins established a Threshold of Toxicological Concern (TTC). A TTC of 2.5 ng/kg body weight per day (BW/d) was set for alternariol (AOH) and alternariol monomethyl ether (AME) and 1500 ng/kg BW/d for tenuazonic acid (TeA) (Arcella et al. 2016). This parameter is suitable for performing exposure assessments and risk characterization when other toxicity data are lacking (Patlewicz et al. 2018). Currently, there is no legislation on these mycotoxins in foods or feeds, but indicative levels for AOH, AME and TeA in certain food were recently established (Commission Recommendation (EU) No. 2022/553). The only *Alternaria* toxin legislated worldwide in a food commodity, at regional level, is TeA, with a limit of 500 µg/kg in sorghum/millet infant food in Bavaria, Germany, based on evidence that the TTC was exceeded by the consumption of this kind of food (Rychlik et al. 2016; Solfrizzo 2017). Further information is imperative to implement safe limits for consumers around the globe. The TTC of 2.5 ng/kg BW/d estimated for both AOH and AME does not consider the possible additive or synergic effect of conjugated forms, such as their sulphates or glycosides (modified *Alternaria* mycotoxins), nor the effect that other co-occurring mycotoxins might have.

Risk assessments are a valuable tool to evaluate the risk to which consumers are exposed (Meerpoel et al. 2021). Exposure assessment can be performed using different approaches. The deterministic approach considers occurrence, consumption, and body weight data as point estimates, using fixed values, while in probabilistic exposure assessments, the variables are described as distributions. In this way, all possible values assumed for each variable are considered and each possible outcome in each scenario is weighted by its occurrence probability (De Boevre et al. 2013; Abdallah et al. 2020). As well, exposure to mycotoxins can be estimated based on occurrence data in food combined with food consumption data (external assessment), or via human biomarkers of exposure in biological samples such as blood or urine (internal assessment) (Meerpoel et al. 2021). When dietary exposure assessments of chemical substances are made on occurrence data, the lower bound (LB) and upper bound (UB) approach is recommended to manage left-censored data (EFSA 2010).

In a risk assessment performed on the European population, data of natural contamination of foods with *Alternaria* toxins showed that the TTC levels for AOH and AME were exceeded in some European countries (Arcella et al. 2016) and vegetarians and toddlers were under greater exposure. Children are of particular concern, being a vulnerable group; they have a higher exposure per kg of BW to contaminants, their enzymatic activity is not fully developed, consequently they have lower ability to break down chemical compounds, amongst other (Boon et al. 2009; Oueslati et al. 2018; Pustjens et al. 2021). Moreover, children may be more sensitive to neurotoxic, endocrine disturbance, and immunological toxic effects up to 4 years old (Huybrechts et al. 2011). This should be of relevance, since alternariols have an estrogenic capacity (Vejdovszky et al. 2017; Dellafiora et al. 2018), and particularly, AOH has androgenic effects (Stypuła-Trębas et al. 2017) that might produce a bigger impact during childhood. Therefore, children should be addressed as a separate group in risk assessments.

So far, there is a lack of data on the occurrence of *Alternaria* mycotoxins in Argentinean products, and, in consequence, no exposure assessment nor risk characterization has been performed on this population. Children are the main target consumers for certain apple by-products such as AJ and apple purees. Therefore, it is crucial to evaluate the incidence of *Alternaria* toxins in clear and cloudy apple by-products, particularly when they are destined for children. Thus, the objectives of this work were: (1) to analyse the natural occurrence of free and modified *Alternaria* mycotoxins in clear and cloudy apple by-products from the Argentinean market and (2) to perform a probabilistic exposure assessment and risk characterisation from the consumption of apple by-products, based on the most relevant *Alternaria* mycotoxins, for the Argentinean population from 6 months to 5 years old.

## Materials and Methods

### Samples

Samples of apple by-products, namely clear and cloudy AJ, and non-clarified apple by-products categorized as apple infant food, and apple marmalades of several commercially available brands were analysed. The samples were randomly collected in stores and supermarkets of Buenos Aires city between 2018 and 2019. A total of 63 samples were analysed: 15 cloudy and 18 clear AJ, 20 infant food and

10 apple marmalades. Blank samples free of *Alternaria* mycotoxin contamination of AJ, infant food and marmalades were purchased in stores in Ghent, Belgium.

## Standards and Reagents

AOH, AME (1 mg standard each), altertoxin-I (ATX-I) and altenuene (ALT) (0.1 mg standard each) were obtained from Fermentek (Jerusalem, Israel) and dissolved in 1 ml of methanol (MeOH). Certified reference standards of TeA and tentoxin (TEN) (101.3 and 100.5 mg respectively, dried down) were obtained from Romer Laboratories Diagnostic GmbH (Tulln, Austria) and dissolved in 1 ml of acetonitrile (ACN). Altertoxin-II (ATX-II) was extracted from *Alternaria alternata* inoculated rice as described previously (Schwarz et al. 2012). Reference standards of conjugated *Alternaria* toxins alternariol-3-sulphate (AOH-3-S) alternariol monomethyl ether-3-sulphate (AME-3-S), alternariol-3-glucoside (AOH-3-G), alternariol monomethyl ether-3-glucoside (AME-3-G) were synthesized as described by Mikula et al. (2013) and stock solutions were prepared at a concentration of 10 µg/ml in MeOH. The internal standard urolithin A (UR-A) (5 mg) was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved in 5 ml of dimethyl sulphoxide (DMSO). The internal standard tenuazonic acid $^{2}$H-13 (1 mg) was bought from Toronto Research Chemical (Toronto, Canada) and dissolved in 1 ml of MeOH. Ultra-pure water was obtained from an Arium® pro system (Sartorius, Goettingen, Germany). ACN (absolute, LC–MS grade) and acetic acid (UPLC/MS) were obtained from BioSolve BV (Valkenswaard, The Netherlands), and ACN (HiPerSolv Chromanorm HPLC grade) was acquired from VWR International (Leuven, Belgium). Sodium chloride (NaCl, 99.9%) was purchased from Merck (Darmstadt, Germany), whereas magnesium sulphate ($MgSO_4$, 99.5%) from Sigma-Aldrich (Bornem, Belgium).

## Determination and Quantification of Alternaria Mycotoxins by LC–MS/MS

The sample preparation was performed using a validated method developed at the Centre of Excellence in Mycotoxicology and Public Health (CEMPH) for the detection and quantification of AOH, AME, ALT, TeA, TEN, ATX-I, AOH-3-S, AOH-3-G, AME-3-S, AME-3-G in apple products (Pavicich et al. 2020a, b; Walravens et al. 2014a, b; Walravens et al. 2016). Additionally, ATX-II was also included in the method.

Determination and quantification of the studied mycotoxins were done on a Waters Acquity UPLC coupled to a XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA) operated in the negative electrospray ionisation ($ESI^{-}$) mode as detailed in Walravens et al. (2014a, b). Two selected reaction monitoring transitions with a specific dwell-time were optimised for each analyte, to increase the sensitivity and the selectivity of the mass spectrometric condition. These are detailed in Supplementary Table 1 as well as the optimized instrumental conditions for ATX-II. The limits of detection (LOD) and quantification (LOQ) for each mycotoxin are informed in Supplementary Table 2.

## Data Treatment

The exposure assessment and risk characterization were done separately for the most relevant *Alternaria* mycotoxins (AOH, AME and TeA) in 3 apple by-products destined for children: clear apple juice, cloudy apple juice, and apple-based infant food. The risk was evaluated for the Argentinean population under 5 years old, divided in two groups: kids from 6 to 23 months old (6–23 MO) and kids from 2 to 5 years old (2–5 YO), because consumption data from by the National Survey of Nutrition and Health of Argentina (MSAL 2012) is divided in these age groups. The occurrence data from the present work for AOH, AME and TeA for apple by-products was used for the exposure assessment and lower (LB) and upper bound (UB) concentration scenarios were constructed with toxins concentrations obtained for the different food matrices. In the LB scenario, the non-detectable values were replaced by 0 and the values below LOQ, by half the LOD value for each compound. For the UB scenario, the non-detectable values were replaced by the LOD and the values < LOQ, by the LOQ as performed by Walravens (2017). These scenarios were then used for the deterministic and probabilistic assessments.

For apple juice (clear and cloudy) the consumption values from the National Survey of Nutrition and Health for fruit juices were used, and for apple infant food, the data for fruit marmalade was used since no specific consumption data for these products was available. The average body weight values for the different age ranges were taken from the Garrahan paediatric hospital (Garrahan 2021).

## Probabilistic Exposure Assessment and Risk Characterisation

The probabilistic exposure assessment was performed using the @Risk® 5.5 add-on for Microsoft Excel. The food intake of the different age groups was modelled using a LogNorm distribution from MSAL (2012) data and the resulting distribution was divided by a Uniform one that considered the weights of each age group. The @Risk function best fit distribution was applied to the LB scenario for each mycotoxin concentration and the resulting distribution was also applied to model the UB scenario. Then, the estimated ng mycotoxin intake by kg BW/d was calculated by first-order Monte Carlo simulation considering 10,000 iterations, multiplying the modelled mycotoxin concentration in each food category by the modelled food intake/kg BW/d. Using the distribution for each food category and age group, the percentage of kids exceeding the TTC values was calculated in the risk characterization.

From the resulting distributions, the number of the specific age groups exceeding the TTC values were calculated multiplying the number reported to consume the food commodity (MSAL 2012) by the percentage exceeding the TTC.

## Results

### Natural Occurrence of Alternaria Mycotoxins in Apple By-Products

The number of positive samples, average concentration, standard deviation, and range of each mycotoxin in clear and cloudy apple juice, apple marmalades and apple infant food is summarized in Table 1.

Clear AJ were contaminated with AME, TeA, TEN, AME-3-S and AOH-3-G in levels above the LOD. From the 18 samples, 13 (72%) were contaminated with at least one mycotoxin, 8 (44%) with only one (AME, AME-3-S, or TEN), 4 (22%) presented co-occurrence of 2, and only 5 (28%) were not contaminated with any of the investigated

**Table 1** Number of positive samples, average concentration, standard deviation, number of samples above LOQ, number of samples above LOD, and range of concentration for each mycotoxin in clear and cloudy apple juices, apple marmalades and apple infant food

| | AOH | AME | ALT | TeA | TEN | ATX-I | AOH-3-S | AME-3-S | AOH-3-G | AME-3-G |
|---|---|---|---|---|---|---|---|---|---|---|
| Clear apple juice | | | | | | | | | | |
| N° of positive samples | 0/18 | 3/18 | 0/18 | 4/18 | 8/18 | 0/18 | 0/18 | 2/18 | 1/15 | 0/15 |
| Average (µg/kg) | n.a | 0.9* | n.a | 13.9 | 2.7* | n.a | n.a | 8.4 | 3.5 | n.a |
| Standard deviation (µg/kg) | n.a | 0.0 | n.a | 10.0 | 0.8 | n.a | n.a | 2.6 | n.a | n.a |
| > LOQ (*n*) | 0 | 0 | 0 | 4 | 2 | 0 | 0 | 2 | 1 | 0 |
| > LOD (*n*) | 0 | 3 | 0 | 4 | 8 | 0 | 0 | 2 | 1 | 0 |
| Range (µg/kg) | < LOD | 0.9*−0.95* | < LOD | 6.2−28.0 | 1.9*−4.1 | < LOD | < LOD | 6.6−10.2 | 3.5 | < LOD |
| Cloudy apple juice | | | | | | | | | | |
| N° of positive samples | 4/15 | 10/15 | 0/15 | 7/15 | 10/15 | 0/15 | 0/15 | 1/15 | 5/15 | 0/15 |
| Average (µg/kg) | 4.5 | 1.5 | n.a | 28.2 | 2.6 | n.a | n.a | 1.5* | 2.1* | n.a |
| Standard deviation (µg/kg) | 1.6 | 0.5 | n.a | 28.0 | 1.1 | n.a | n.a | n.a | 0.8 | n.a |
| > LOQ (*n*) | 4 | 7 | 0 | 6 | 2 | 0 | 0 | 0 | 3 | 0 |
| > LOD (*n*) | 4 | 10 | 0 | 7 | 10 | 0 | 0 | 1 | 5 | 0 |
| Range (µg/kg) | 2.2−6.2 | 0.9*−2.2 | < LOD | 3.0*−79.8 | 1.2−4.6 | < LOD | < LOD | 1.5* | 1.1*−2.9 | < LOD |
| Apple marmalades | | | | | | | | | | |
| N° of positive samples | 0/10 | 4/10 | 0/10 | 1/10 | 1/10 | 0/10 | 0/10 | 0/10 | 4/10 | 0/10 |
| Average (µg/kg) | n.a | 4.3 | n.a | 144.3 | 92.4 | n.a | n.a | n.a | 13.3 | n.a |
| Standard deviation (µg/kg) | n.a | 0.5 | n.a | n.a | n.a | n.a | n.a | n.a | 2.7 | n.a |
| > LOQ (*n*) | 0 | 4 | 0 | 1 | 1 | 0 | 0 | 0 | 4 | 0 |
| > LOD (*n*) | 0 | 4 | 0 | 1 | 1 | 0 | 0 | 0 | 4 | 0 |
| Range (µg/kg) | < LOD | 3.9−5.0 | < LOD | 144.3 | 92.4 | < LOD | < LOD | < LOD | 10.3–16.8 | < LOD |
| Apple infant food | | | | | | | | | | |
| N° of positive samples | 7/20 | 20/20 | 0/20 | 14/20 | 19/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| Average (µg/kg) | 5.0 | 6.5 | n.a | 48.9 | 30.7 | n.a | n.a | n.a | n.a | n.a |
| Standard deviation (µg/kg) | 4.4 | 2.2 | n.a | 60.7 | 20.4 | n.a | n.a | n.a | n.a | n.a |
| > LOQ (*n*) | 2 | 20 | 0 | 14 | 18 | 0 | 0 | 0 | 0 | 0 |
| > LOD (*n*) | 7 | 20 | 0 | 14 | 19 | 0 | 0 | 0 | 0 | 0 |
| Range (µg/kg) | 1.7*−13.7 | 4.4−14.7 | < LOD | 6.5−225.7 | 4.1−92.2 | < LOD | < LOD | < LOD | < LOD | < LOD |

*AOH* alternariol, *AME* alternariol monomethyl ether, *ALT* altenuene, *TeA* tenuazonic acid, *TEN* tentoxin, *ATX-I* altertoxin-I, *AOH-3-S* alternariol-3-sulphate, *AME-3-S* alternariol monomethyl ether-3-sulphate, *AOH-3-G* alternariol-3-glucoside, *AME-3-G* alternariol monomethyl ether-3-glucoside, Average, standard deviation and range were calculated over positive samples. *n.a.* not applicable

*Values between LOD and LOQ

*Alternaria* mycotoxins. TEN was the most prevalent mycotoxin, found in 8 (44%) samples.

In cloudy AJ, the concentration and prevalence of *Alternaria* mycotoxins was higher than in the clear ones, except for the modified toxins AME-3-S and AOH-3-G. All the mycotoxins found in clear AJ were detected in the cloudy ones, plus AOH. From the 15 samples, 14 (93%) were contaminated with at least one *Alternaria* mycotoxin, 3 (20%) presented concentrations above the LOD for 1 mycotoxin only, 5 (33%) had co-occurrence of 2 mycotoxins, 1 (7%) with 3, 4 (27%) with 4, and one sample (7%) was simultaneously contaminated with 5 mycotoxins. The predominant toxins in cloudy AJ were AME and TEN, present in 67% samples.

Apple marmalades were contaminated with AME, TeA, TEN, and AOH-3-G in levels above the LOQ. From the 10 samples, 7 (70%) were contaminated with at least one mycotoxin, 5 (50%) with only 1, one (10%) presented co-occurrence of 2, and one (10%) of 3 mycotoxins. Only 3 (30%) samples were not contaminated with any of the investigated *Alternaria* mycotoxins. AME and AOH-3-G were the most frequent mycotoxins in marmalades, (40%).

For apple infant food, the concentration and prevalence of *Alternaria* mycotoxins was higher than in the marmalades, except for AOH-3-G, which was not detected in infant food. As well, the prevalence and range were higher than for clear and cloudy AJ. From the 20 samples, all were contaminated with at least 2 *Alternaria* mycotoxins, 8 (40%) presented concentrations above the LOD for 3 mycotoxins, and 6 (30%) had co-occurrence of 4 mycotoxins. The most frequent toxins in apple infant food were AME and TEN, with a 100% and 95% prevalence respectively, followed by TeA (70%) and AOH (35%).

## Probabilistic Exposure Assessment and Risk Characterisation for Apple Juices

The best fitted distribution for the concentration of AOH in cloudy AJ, and AME and TeA in both clear and cloudy AJ, was exponential. The probabilistic exposure could not be calculated for AOH in clear AJ since all samples had concentrations below the LOD; thus, values were 0 in the LB scenario and equal to LOD in the UB scenario.

Supplementary Table 3 shows the estimated intake of mycotoxins by the different age groups through the consumption of clear and cloudy AJ in the LB and UB scenarios. The mean and P95 estimations were taken from the corresponding distributions. Table 2 shows the percentage of 6 to 23-month-old, and 2 to 5-year-old Argentinean kids exceeding the TTC for AOH, AME and TeA calculated from the distributions.

**Table 2** Percentage of Argentinean kids from 6 to 23 months old or 2 to 5 years old exceeding the Toxicological Threshold of Concern (TTC) of alternariol, alternariol monomethyl ether and tenuazonic acid from clear and cloudy apple juice in the lower bound (LB) and upper bound (UB) scenarios

| Mycotoxin | Food category | Age group | % Exceeding TTC | |
|---|---|---|---|---|
| | | | LB | UB |
| AOH | Clear AJ | 6–23 MO | n.a | n.a |
| | | 2–5 YO | n.a | n.a |
| | Cloudy AJ | 6–23 MO | 25.4 | 87.3 |
| | | 2–5 YO | 24.7 | 86.3 |
| AME | Clear AJ | 6–23 MO | 0.4 | 59.3 |
| | | 2–5 YO | 0.2 | 56.6 |
| | Cloudy AJ | 6–23 MO | 50.2 | 76.9 |
| | | 2–5 YO | 48.8 | 75.4 |
| TeA | Clear AJ | 6–23 MO | 0 | 0 |
| | | 2–5 YO | 0 | 0 |
| | Cloudy AJ | 6–23 MO | 0.7 | 1.5 |
| | | 2–5 YO | 0.3 | 0.8 |

*AOH* alternariol, *AME* alternariol monomethyl ether, *TeA* tenuazonic acid, *6–23 MO* Argentinean kids from 6 to 23 months old, *2–5 YO* Argentinean kids from 2 to 5 years old, *n.a.* not applicable

## Probabilistic Exposure Assessment and Risk Characterisation for Apple Infant Food

The best fitted distribution for mycotoxin concentration in infant food was exponential for AOH and TeA, and LogLogistic for AME. Supplementary Table 4 summarizes the exposure to mycotoxins through apple infant foods in the different age groups. Table 3 shows the percentage of kids exceeding the corresponding TTC values for each mycotoxin.

The number of kids at risk is summarized in Table 4. For juices, 19,474 kids from 6 to 23 MO and 73,189 kids from 2 to 5 YO are reported to consume these products. Because the population has been growing, and the consumption has not been updated, the values for the UB scenario for each product and mycotoxin were used. For clear AJ, only AME represented a risk and 11,548 kids from 6 to 23 MO and 41,425 kids from 2 to 5 YO would be exposed to this mycotoxin in levels above the TTC. From consuming cloudy AJ, a total of 17,001 kids between 6 and 23 MO and 63,162 children from 2 to 5 YO would exceed the TTC of AOH, and 14,976 for the 6–23 MO group, and 55,185 from the 2–5 YO for AME. A much lower number of children would be exceeding the TTC of 1500 ng/kg BW d for TeA by consuming cloudy AJ; they amounted 292 and 586 kids from 6–23to 2–5 YO, respectively.

Since apple based infant food was first available in the country long after the survey took place, unfortunately

**Table 3** Percentage of Argentinean kids from 6 to 23 months old or 2 to 5 years old exceeding the toxicological threshold of concern (TTC) of alternariol, alternariol monomethyl ether and tenuazonic acid through apple infant food in the lower bound (LB) and upper bound (UB) scenarios considering consumption data from MSAL (2012) and the estimated consumption of the whole pack of 90 g

| Mycotoxin | Food category | Age group | % Exceeding TTC (MSAL consumption) | | % Exceeding TTC (complete pack consumption) | |
|---|---|---|---|---|---|---|
| | | | LB | UB | LB | UB |
| AOH | Apple infant food | 6–23 MO | 8.0 | 47.2 | 28.6 | 86.6 |
| | | 2–5 YO | 6.3 | 39.7 | 24.2 | 81.1 |
| AME | Apple infant food | 6–23 MO | 91.2 | 91.3 | 95.5 | 95.8 |
| | | 2–5 YO | 84.9 | 85.4 | 95.5 | 95.8 |
| TeA | Apple infant food | 6–23 MO | 0 | 0 | 0.2 | 0.4 |
| | | 2–5 YO | 0 | 0 | 0 | 0 |

*AOH* alternariol, *AME* alternariol monomethyl ether, *TeA* tenuazonic acid, *6–23 MO* Argentinean kids from 6 to 23 months old, *2–5 YO* Argentinean kids from 2 to 5 years old

**Table 4** Number of Argentinean kids from 6 to 23 months old or 2 to 5 years old exceeding the toxicological threshold of concern (TTC) of alternariol, alternariol monomethyl ether and tenuazonic acid from consuming clear and cloudy apple juice (AJ) and apple infant food in the upper bound scenario considering consumption data from MSAL, (2012) for juices and the consumption of the whole apple infant food pack of 90 g

| Food category | Age group | Number of children exceeding the TTC | | |
|---|---|---|---|---|
| | | AOH | AME | TeA |
| Clear AJ | 6–23 MO | 0 | 11,548 | 0 |
| | 2–5 YO | 0 | 41,425 | 0 |
| Cloudy AJ | 6–23 MO | 17,001 | 14,976 | 292 |
| | 2–5 YO | 63,162 | 55,185 | 586 |
| Apple infant food | 6–23 MO | 22,716 | 25,126 | 105 |
| | 2–5 YO | 175,093 | 206,830 | 0 |

*AOH* alternariol, *AME* alternariol monomethyl ether, *TeA* tenuazonic acid, *6–23 MO* Argentinean kids from 6 to 23 months old, *2–5 YO* Argentinean kids from 2 to 5 years old

no consumption data were accessible. Hence, the consumption data for fruit marmalades was used as it was the most similar product, although it was a rough underestimation. The percentage of kids exceeding the TTC was calculated considering the complete 90 g pack consumption. The total of children from 6 to 23 MO consuming these products were 26,228, and the children from 2 to 5 YO were 215,898. Therefore, the number of children from 6–23 to 2–5 YO surpassing the TTC of AOH from the sole consumption of apple infant food were 22,716 and 175,093 kids from 6–23 to 2–5 YO, respectively. For AME, over 91% and 85% exceeded the TTC, representing 25,126 and 206,830 kids. For TeA, only 0.4% of kids in the UB and consuming the whole pack of infant food and from the 6–23 MO age group exceeded the TTC, representing 105 kids.

## Discussion

### Natural Occurrence of Alternaria Mycotoxins in Apple Juices

A wide variety of *Alternaria* toxins occurred in clear and cloudy apple juices. TEN was the most frequent mycotoxin, although in low levels. In our previous study the clarification step was effective to reduce the concentration of this mycotoxin (Pavicich et al. 2020a, b). Thus, its presence in commercial clear juices suggests the use of low-quality raw material for their production. Other studies also reported TEN in low frequencies (Li et al. 2020) or traces (Zwickel et al. 2016) in apple juices.

TeA was the second most prevalent mycotoxin in clear AJ (22%) and the one with the highest mean concentration. Likewise, it was the only mycotoxin present in the clear apple concentrates analysed by Pavicich et al. (2020a, b). Studies from China and Europe also found TeA in apple juice, and usually in higher concentrations than the other *Alternaria* mycotoxins (Gross et al. 2011; Prelle et al. 2013; Fan et al. 2016; Walravens et al. 2016; Zwickel et al. 2016; Li et al. 2020).

AME was detected in 3 in levels between LOD (0.3 μg/kg) and LOQ (1.3 μg/kg) in clear AJ. From several studies, AME was only detected in AJ in China at low concentrations as well (Fan et al. 2016; Li et al. 2020). The modified forms AME-3-S and AOH-3-G were found for the first time in commercially available AJ. The glucosyl forms are believed to be detoxification mechanisms of the plants, while the sulphonated ones can also be produced by the fungi (Puntscher et al. 2020).

The same mycotoxins were detected in cloudy AJ (AME, TeA, TEN, AME-3-S and AOH-3-G) with the addition of AOH. However, their concentration and frequency were higher. This is in accordance with our previous results, in which the cloudy concentrates were significantly more contaminated than the clear ones. When cloudy apple concentrates were analysed, AOH, AME, TeA, and TEN were present (Pavicich et al. 2020a, b). Consistently, the same mycotoxins were found in commercial cloudy AJ, TEN and AME being the most frequent ones and TeA the toxin present at the highest concentration. These results suggest that the dilution from concentrate to commercial product is not sufficient to reduce these mycotoxins to non-detectable levels. A few studies from Europe and China analysed *Alternaria* mycotoxin contamination of apple juices, but they did not specify if the samples were clear or cloudy AJ, and some of them did not find samples with levels above LODs (López et al. 2016).

## Natural Occurrence of Alternaria Mycotoxins in Apple Purees

The concentration and prevalence of mycotoxins in apple purees was higher than those from the juices. Apple marmalades were contaminated with AME, TEN, TeA, and AOH-3-G, AME being the most frequent and TeA the one found at the highest concentrations. Apple infant foods were contaminated with AOH, AME, TEN and TeA, and again AME was the most frequent being present in all the tested samples. The concentration and prevalence were higher in the apple infant food than in any other apple by-product, raising particular concern since infants are a vulnerable group. Few studies investigated the presence of these mycotoxins in non-clarified apple products. In China, AME and TEN were present in apple jam (Li et al. 2020) and a study by Ackermann et al. (2011) found AOH in all the apple sauce samples studied ($n=10$). More recently, Gotthardt et al. (2019) found AOH, AME, TEN and TeA in infant foods. However, no occurrence data from Argentina was available previous to this study. This information is crucial to assess the consumer's risk.

In line with a recent Austrian study (Braun et al. 2021), no modified forms of *Alternaria* mycotoxins were detected in infant foods. On the other hand, AOH-3-G was found in the marmalades, but its parent form was not found. More information about the modification of *Alternaria* mycotoxins is needed and the toxicity of these compounds should also be tested, in order to perform more accurate risk assessments.

## Risk Characterization

Based on the data generated, it was possible to characterize the risk associated to the presence of AOH, AME and TeA in apple products for children in Argentina for the first time., However, the model had limitations, the lack of updated consumption data could lead to biased results and changes in the consumption pattern might have a strong impact on the exposure calculations and should be further evaluated. Clear and cloudy AJ were assumed to have the same consumption in this assessment since no distinction was made in the MSAL (2012). On the other hand, apple infant food was only recently available in the local market; therefore, approximations had to be made to estimate the risk, sacrificing precision. As well, a bigger sample size is advisable, with periodic follow up of mycotoxin concentrations. No other occurrence data of these products were available and information of *Alternaria* mycotoxin contamination in Argentina is scarce for most food products. Another factor of uncertainty in the model was derived from samples with left-censored data points. Therefore, the LB and UB scenario provided a best- and worst-case scenario estimation (EFSA 2010).

For all the food commodities analysed, the highest risk was associated with the alternariols, which have estrogenic potential. AME posed a risk from the consumption of clear and cloudy AJ, and AOH only from cloudy AJ, where by far exceeded the TTC of 2.5 ng/kg BW/d for the different age groups. The daily intake represented 2 to 57 times the TTC value and 87.3% (17,001) and 86.3% (63,162) of consumers of cloudy AJ were exposed to higher levels of AOH compared to the TTC. For AME, the TTC value was exceeded in all the proposed scenarios from consuming cloudy AJ, surpassing the TTC value from 3 to 27 times exposing 79.9% (14,976) and 75.4% (55,185) of children. For TeA, even though the TTC was not exceeded in any of the deterministic scenarios, the consumption of cloudy AJ represented from 8 to 62% of the total TTC value for both age groups.

Overall, a higher risk was found for non-clarified products. Particularly, regardless the concentration scenario or consumption of apple infant foods, over 90% of children between 6 and 23 MO and 84% of children between 2 and 5 YO are exposed to levels above the TTC of AME, raising additional concern. When a more realistic consumption (whole pack) was tested, the TTC of AOH was exceeded 3 to 10 times for both age groups. In the worst-case scenario 86.6% (22,716) and 86.1% (175,093) of the consumers from 6–23 to 2–5 YO, respectively, exceeded the TTC.

Even though TeA was found at the highest concentrations among all the products, it did not pose a high risk for children consuming apple products. However, this result does not consider the intake of TeA from other food commodities and the contribution that other foods might have on the intake of AOH and AME should not be overlooked as well as. Other food commodities in Argentina have been reported as contaminated with AOH, AME and TeA, such as vegetables or cereals, usually mixed with fruits to obtain other infant foods (Azcarate et al. 2016; Castañares et al. 2020; da Cruz Cabral et al. 2016; Romero Bernal et al. 2019; Terminiello et al. 2006). The data generated here should be incorporated to a comprehensive, more holistic, risk characterisation through a total diet study in Argentina. As well, modified mycotoxins should also be considered in risk assessments, since modified forms of alternariols have been found in apple by-products and they may hydrolyse in the digestive tract to form unconjugated mycotoxins (Chen et al. 2021); thus, their potential toxicity could be equivalent to that of alternariols.

Even though some studies evaluated the risk from exposure to *Alternaria* mycotoxins in the European population (Arcella et al. 2016; Sprong et al. 2016; Vin et al. 2020), and China (Zhao et al. 2015), the occurrence data and consumption patterns differ from the Argentinean perspective. Nevertheless, data of natural contamination of foods with *Alternaria* toxins showed that the TTC levels for AOH and AME were exceeded in some European countries (Arcella et al. 2016), as well as in China (Zhao et al. 2015). A recent total diet study in the Netherlands showed that the TTC value for the alternariols was exceeded for kids from 1 to 2 YO, and that apple infant foods and fruit juices were responsible for the high intake of AOH (Pustjens et al. 2021). Therefore, the inclusion of apple by-products in the current Commission recommendation (EU 2022/553) on indicative levels for *Alternaria* mycotoxins should also be addressed.

Considering the estrogenic potential of alternariols (Vejdovszky et al. 2017; Dellafiora et al. 2018), the combinatory toxic effects they may exert (Fernández-Blanco et al. 2016), and the overall vulnerability of children, the numbers here presented should raise attention. The impact that early-life exposure to these mycotoxins could imply on the (Argentinean) population should be considered, reflecting to the estrogenic post-puberty effects. Taking into account that other food commodities alone may pose a risk for consumers from *Alternaria* contamination, a holistic assessment such as a total diet study or human biomonitoring in infants in Argentina is warranted. *Alternaria* mycotoxins are no longer emerging (Aichinger et al. 2021), and their presence in food commodities, especially when they are intended for infants should be addressed by implementing not only increased prevention and controls measures by producers, but also implementation of regulation by both national and international authorities.

## Conclusions

To our knowledge, this is the first report of *Alternaria* mycotoxins and their modified forms in commercial apple by-products from the Argentinean market. All the different food categories analysed were contaminated with *Alternaria* toxins, but higher levels were found in non-clarified products destined to infants, implying a potential risk associated to these products.

The risk of exposure to *Alternaria* toxins from consuming apple by-products was characterized for children, showing a high exposure to alternariols and a greater risk associated to non-clarified products, particularly infant food. Even though the risk posed for TeA was low in the present analysis, it should not be underestimated, since this mycotoxin was found in higher concentrations and is broadly distributed in other food commodities.

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**Data Availability** The datasets analysed during the current study are available in the Biblioteca Virtual de Nutrición repository, https://cesni-biblioteca.org/ennys2/.

## Declarations

**Competing interests** The authors have no relevant financial or non-financial interests to disclose.



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